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JIMR - Journal of Islamic Medicine Research
Published by Faculty of Medicine, Universitas Islam Malang
Email: jimr@unisma.ac.id
Home Page : http://riset.unisma.ac.id/index.php/fk
E-ISSN: 2580 927X
Pages: 42 – 50

The growth rate of *S. aureus* when treated with Azithromycin alone and combined with *Syzygium cumini* decoctation

Rio Risandiansyah*, Yoni Rina Bintari**
*Departement of Microbiology, Faculty of Medicine, Islamic Malang University
**Departement of Bio Chemistry, Faculty of Medicine, Islamic Malang University
Email: riorisandiansyah@unisma.ac.id

ABSTRACT

Introductiom*: Resistance to antibiotics is the result of adaptive and spontaneous mutations, which can be suppressed by use of combinatory antibiotics. Synergistic effects between certain plants in combination with antibiotics is known, yet the mutation frequency of such combinations is still unexplored. Therefore, this study aims to measure the spontaneous mutational frequency of S. aureus for Azithromycin (AZM) resistance alone and in combination with Syzygium cumini decocta (SCD), known for its antimutagenic effect.*

Method: *This study uses fluctuation analysis with 39 replicate cultures in selective media using AZM alone, SCD alone, and AZM combined with SCD at 1x MIC and analysing resistant strains based on its log phase delay in liquid media in a 24-hour period. AZM and SCD showed antibiotic activity against S. aureus.*

Results: *Time-point growth comparison showed two cultures in AZM selective medium with no delayed log phase between S. aureus in non-selective medium and absorbance >1.0 after 24 hours, indicating resistance to AZM.*

Conclusion: *Increased growth suppression was observed in combination between AZM combined with SCD, compared to AZM and SCD alone. The spontaneous mutation frequency of S. aureus against AZM was 0.182, while both SCD and AZM combined with SCD had zero mutational events for antibiotic resistance.*

Keywords: *Antibiotic Resistance, Combinatorial Antibiotic Therapy (CAT), Mutation frequency, Syzygium cumini.*

INTRODUCTION

The increase of antibiotic resistance incidence, both globally and in several hospitals in Indonesia, raises some concerns that the world is heading to an antibiotic crisis, or even returning to the pre-antibiotic era [1–3]. Indonesia's high population, low sanitation, and low education levels in several regions creates places vulnerable to communicable diseases, making infection as among the highest cause of death in Indonesia [4]. However, aside from few information from various hospitals, sufficient national antibiotic resistence data is still lacking.

Several theories attempt to explain the mechanism of how antibiotic resistance occurs. From an epidemiological stand point, studies correlate the misuse or incorrect prescription of antibiotics with increased antibiotic resistance [5,6], creating a condition where only sublethal concentrations of the antibiotics is received. The use of antibiotics in agricultural industry (i.e, to increase weight gain of farm animals), are also shown to increase overall antibiotic

resistance [6–8]. One solution for increasing resistance, as proposed by WHO, is the increasing the effectiveness of current antibiotics [9].

Research has shown that a synergistic effect exists between certain plant extract when combined with antibiotics, shown by an increase of antibiotic efficacy [10]. Also, synergistic effects may also be found in plants not having antibacterial effects by itself and may even increase the effectiveness of a drug against resistant strains [11]. This may indicate that the use of certain plant extracts in combination with antibiotics can be used in therapeutical regiments for infections with resistant pathogens, however, the effectiveness of plants in Indonesia to be used in conjunction with antibiotics has not yet been explored.

This preliminary study aims to measure the effectiveness of antibiotics with a plant extract with antibiotic properties. This study uses *Staphylococcus aureus* as it is a well studied organism which is usually a subject in antibacterial screening, and is prominently found in nosocomial and community based infections. Similarly, Azithromycin was selected due to its use in outpatient treatments. Lastly, this study uses *Syzygium cumini* as the herbal crude extract subject. *Syzygium cumini* has been shown to have antibacterial activity (including against *S. aureus*) [12], and antifungal activity against both sensitive and resistant *Candida* strains [13,14].

MATERIAL AND METHODS

Sample sources used in this study

Syzygium cumini simplisia was obtained from Batu Materi Medika (BMM), Jawa Timur, Indonesia, purchased early January 2017. The simplisia was stored in a closed container at room temperature which was not exposed to direct sunlight. Azithromycin (AZM) used was a tablet (500 mg) which was then crushed and mixed with 100 ml distilled H_2O, and stored in cold temperature until further use. *Staphylococcus aureus* strains was a clinical isolate, isolated by the Microbiology Department, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.

From slants, *S. aureus* was inoculated on Nutrient Agar (NA) (HiMedia Laboratories®; composition: Peptic digest of animal tissue 5 g/L, NaCl 5 g/L, Beef extract 1.5 g/L, Yeast extract 1.5 g/L, Agar 15 g/L), and cultivated in Nutrient Broth (NB) (HiMedia Laboratories®; composition: Peptone 10 g/L, Beef extract 10 g/L, NaCL 5 g/L). For each inoculation, *S. aureus* cultures were incubated at 37°C for 18 to 24 hours, and stored in 4-8°C until further use. Determination of bacterial number (CFU/ml) was conducted using a Miles and Misra method.

Decoctation protocol of *Syzygium cumini*

Decoctation was conducted with a simplisia to distilled water ratio of 1:10 (w/v) at 90°C for 30 minutes. Yield was measured after heating at 60°C. *Syzygium cumini* decocta (SCD) stocks were made to a 100 mg/ml concentration and stored in cold temperature until further use.

Phytochemical content determination from *Syzygium cumini* decocta (SCD)

The phytochemical content determination was conducted on obtained SCD at 100 mg/ml for alkaloid, terpenoids, phenolics, and saponin, using Mayer and Dragendroff, H_2SO_4, $FeCl_3$ assay, and foam test, respectively.

Determination of antibacterial activity against *S. aureus*

Antibacterial activity was determined using an agar diffusion method. *S. aureus* was first made into an OD_{625nm} 0.2 in liquid stocks (calculated using Miles and Misra method to be ≈6.06 x 10^{10} CFU/ml), inoculated at 1% (v/v) of medium. Test samples were inserted into each 6 mm wells, and the plates incubated at 37°C for 18 – 24 hours. The clear zone surrounding each well was measured using a ruler. Colistin (COL) was used as a bacterial control.

MIC was determined using a macrodilution method of at least four sample concentrations that did not show inhibition on previous antibacterial activity testing. Briefly, extracts were tested in serial dilutions by mixing 1 ml of extract with the same volume of nutrient broth in a reaction tube. One ml was then taken out of that reaction tube and transferred into a new tube containing the same volume of nutrient broth, and this process repeated until five or six serial dilutions was achieved. Finally, one ml of a bacteria suspension at 10^5 CFU/ml was pipetted into each tube. The reaction tubes were then incubated at 37°C for 20 to 24 hours, and observed for turbidity. A control containing nutrient broth only, and a control containing bacterial suspension (with no extract) was also made to act as comparison.

Growth rate analysis of *S. aureus* in different selective medium

RESULTS AND DISCUSSION

Firstly, stock bacteria were first diluted to obtain approximately 10^5 CFU/ml, which would be the initial inoculation. Cultures were then transferred into 39 1.5 ml microtubes, each containing 100 µl of the initial inoculation cultures. The microtubes were incubated for 18 – 20 hours at 37°C.

After incubation, each microtubes were transferred into two wells in a 96-well plate in one control group and three treatment groups. The control ('non-selective') group, composed of 6 wells, contains only nutrient broth (at 4 times concentration, to refresh the medium components) and sterile distilled water. The treatment group (each group was composed of 24 wells) had similar composition with the control group, however, instead of sterile distilled water, Azithromycin (AZM), *Syzygium cumini* decocta (SCD) or a combination of AZM and SCD was added at 1 MIC. Blanks were then added (Nutrient broth without bacteria).

To measure and observe growth rate, the plates were then measured in an EPOCH Spectrophotometer set at 37°C, and read at a wavelength of 600 nm at each hour for 24 hours. The resulting absorbance were then plotted to be measured for lag, log and stationary times, as well as maximal growth.

Data analysis

Absorbance readings acquired from the spectrophotometer was first detracted with its the mean value of its corresponding blanks. Growth rate was obtained by using Gen5TM software. Box-plot and Box and whiskers diagram for growth rate analysis of the bacteria was created using Microsoft Excel, by measuring mean, median, maximum, minimum, 25% and 75% quartiles.

Phytochemical analysis of Syzygium cumini decocta (SCD) showed the presence of alkaloids, terpenoids, phenolics, and saponins

Phytochemical analysis of SCD qualitatively showed positive results for alkaloids, terpenoids, phenolics and saponin. These findings do not contradict other research done in this area, based on

phytochemical reviews on *Syzygium cumini* [15].

Syzygium cumini decocta (SCD) and Azithromycin (AZM) showed activity against _S. aureus_

Both SCD and AZM showed activity against *S. aureus*, and clear zone diameter measurement, indicating antibacterial activity, was displayed in table 1. SCD had an activity of 14 mm at its highest concentration. AZM, at 5 mg/ml, had an activity of 20 mm, whilst COL had no visible activity against S. aureus. As the wells used in this experiment had a size of 6 mm, no visible clear zones could only be assessed as smaller than the well size (< 6mm) and therefore not indicative of no activity.

Table 1. Zone of Inhibition (ZOI) measurement on _S. aureus_ against SCD and AZM

Sample tested	Conc. (mg/ml)	ZOI (mm)
***Syzygium cumini* decocta**	100	14
	50	12
	25	10
	12.5	8
	6.25	< 6
	3.125	< 6
	1.5625	< 6
	0.78125	< 6
Azithromycin	5	20
Colistin	5	< 6

From this experiment, it can be confirmed that SCD possessed antibiotic activity against *S. aureus* as well as the AZM used. Previous research has also found antibacterial activity potential of *Syzygium cumini*, in ethanolic [12] or methanolic extracts and in its essential oils [16]. However, another study reports that no activity was found in aqueous extracts [17]. Therefore, this study reports activity of SCD obtained from Indonesian sources against *S. aureus*. The MIC was determined to be 1/64 times dilution from stock, or 1.5625 mg/ml for SCD, and 0.039 mg/ml or 39 mg/L for AZM (data not shown), and this concentration was used in further experiments.

Growth analysis of 24-hour S. aureus inoculum showed normal growth in non-selective media and inhibition in all selective media

The growth rate of *S. aureus* during the selection phase in control medium and treatment groups is shown in Figure 1. Growth curve of *S. aureus* in the control group ('non-selective' medium) shows a normal growth pattern, in which the lag phase was observed to be less than 1 hour, the exponential or log phase occurs until the a 8 – 9 hours of incubation, and followed by a stationary phase (Figure 1). The initial culture (at time 0) had a median optical density of 0.229 (Q1 = 0.202, Q3 = 0.252, Mean±SD = 0.224±0.045) and after 24 hours reached 1.5 (Q1 = 1.486, Q3 = 1.516, Mean±SD = 1.5±0.028). Low variance of data was observed in the results, showing high consistency between replicates.

On all treatment groups, bacteria inoculum growth after 24 hours was inhibited. Based on its median growth, bacteria on AZM alone showed the highest median of all treatment groups, with a 24-hour growth of 0.539 (Q1 = 0.418, Q3 = 0.672, Mean±SD = 0.573±0.263). However, high variance was observed. The lowest growth on S. aureus treated with SCD and AZM combination, in which the 24-hour growth was 0.302 (Q1 = 0.245, Q3 = 0.339, Mean±SD = 0.305±0.095), followed by treatment with SCD alone, of 0.431 (Q1 = 0.341, Q3 = 0.512, Mean±SD = 0.430±0.114).

AZM, like most macrolides, has bacteriostatic activity [18,19], and acts inhibiting protein synthesis, as well as preventing the formation of biofilm by the inhibition of quorum sensing molecules [20]. Based on the growth curve (Figure 1), findings in this study shows similar patterns of bacteriostatic activity as described in other studies [21]. Similarly, while the active compounds of SCD was not elucidated in this study, based on the same growth patterns, it can be speculated that SCD also possess bacteriostatic activity.

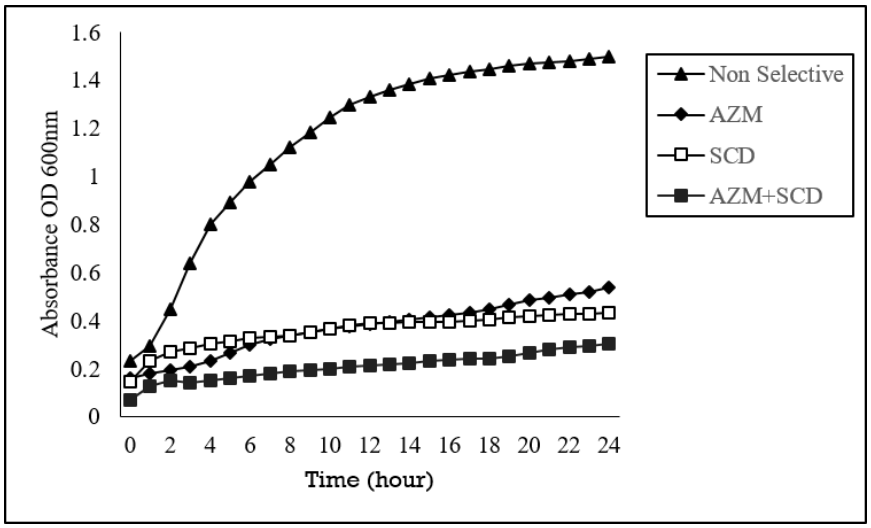

Figure 1. Median of growth of *S. aureus* in non-selective and selective media (AZM, SCD and AZM+SCD), showing decreased growth in selective medium.

Growth rate analysis suggest that lag extension occurs in several cultures in different treatment groups

To analyze maximum growth rate, growth of *S. aureus* based on its absorbance changes were analyzed in time blocks corresponding to normal growth phases. This would allow the visualization of small but consistent increase in growth rate, as well as sudden peaks in growth rate in a certain period. Normal growth, in this case, consists of a one-hour long lag phase, followed by an 8-hour log phase, and entering stationary phase until the 24th hour mark. Therefore, the time blocks created for analysis was 0 to 1-hour time block (corresponding to lag phase), 1 to 4-hour time block (corresponding to lag to log transition, and early log phase), 4 to 8-hour time block (corresponding to late log phase and log to stationary transition), 8 to 12 -hour, 12 to 16-hour, and 16 to 24-hour (each corresponding to stationary phase).

In this study, several cultures in each treatment group showed different time periods for maximum growth rate in different selective media (Figure 2). In AZM alone selective medium, several cultures showed growth rate of over 0.1 absorbance in different time periods. In

13/24 cultures, such growth rate was observed between 4 – 8 hours, in which 10 had its maximum growth rate in that period. Between 16 – 24 hours, 5/24 cultures had high growth rate in which 4 cultures was its maximum growth rate. Two cultures did not show any delay in its lag phase (based on inoculum growth in non-selective medium), with a maximum growth rate between 1 – 4 hours.

At a lesser degree, this was also observed on SCD selective medium. High growth rate was observed on 6/25 cultures, with 2 cultures had its maximum growth rate between 0 – 1 hours, 2 at 1 – 4 and another 2 at 4 – 8 hours post-inoculation. High growth rate was not observed after 16 hours, however, growth pattern of inoculum in AZM+SCD selective medium, however, high growth in any time periods were not observed.

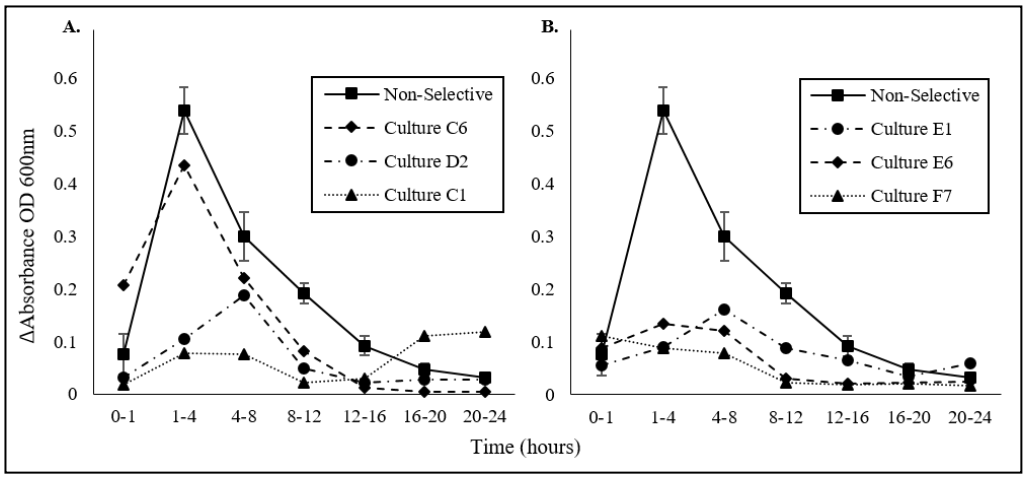

Figure 2. A. Growth patterns of *S. aureus* inoculum in AZM selective medium, when compared to growth in non-selective medium, in which representatives of periods with the highest growth (>0.1) was shown (Culture C6 for between 1 to 4 hours, D2 for between 4 to 8 hours, and C1 for between 16 – 20 hours and 20 – 24 hours). B. Similarly, growth patterns of inoculum in SCD alone selective medium (Culture E1 for between 1 to 4 hours, E6 for between 4 to 8 hours, and F7 for between 20 – 24 hours). No cultures in AZM combined with SCD had growth >0.1.

Growth kinetics has been shown to be able to determine bacterial susceptibility to antibiotics. Several research show that antibiotic resistance confers little to no fitness cost to the bacteria [22,23], and therefore having no impact on growth rate. Furthermore, bacteria susceptible to antibiotics was known to have a prolonged lag phase (or a delayed log phase), in which the log phase may emerge after 12 to 24 hours after inoculation [24], and this prolonged lag phase may indicate that the bacteria resume growth after the removal of the antibiotic [25].

While AZM selective medium showed several bacteria with the potential with survive its bacteriostatic treatment, combination between SCD and AZM suppresses the growth of *S. aureus* further, with no growth over 0.1 in 4-hour periods was observed. Therefore, the combination between SCD and AZM in this case may be used in synergistic treatment of *S. aureus*.

CONCLUSION

Delayed log phase was observed in 13/24 *S. aureus* inoculum under selective AZM medium, under SCD medium 6/24 cultures was observed, and 0/24 was

observed under AZM with SCD combination, indicating possible synergistic action.

FUTURE DIRECTIONS

Further tests may be required to determine the exact nature of *S. cumini* decocta-azythromycin interaction to determine the Chou-Talalay theorem combination index (CI) as described in previous research [26]. Studies in the relationship between lag phase extension, as found in this study, and chance for resistance is required as well as whether resistance against plant extracts is possible is also required.

ACKNOWLEDGEMENT

We would like to thank Fakultas Kedokteran Universitas Islam Malang, for providing the funding and laboratory equipment for this study.

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